EXHIBIT 43

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Expression of Ribosomal DNA Insertions in Drosophila melanogaster

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Summary

Approximately half of the ribosomal genes on the X chromosome of Drosophila melanogaster are interrupted by an insertion of type 1. Nuclear RNA from D. melanogaster embryos was transferred to DBM paper and hybridized with cloned type 1 insertion sequences. With a DNA fragment derived specifically from large insertions, transcripts were detected between 5 and 10 kb. These insertion transcripts represent less than one RNA molecule per nucleus, which is more than three orders of magnitude below the concentration of nascent rRNA chains, as determined by kinetics of hybridization. With a DNA fragment derived from the right end of large insertions which is also complementary to short insertions, more discrete RNA bands appeared with sizes between 1 and 8.5 kb, representing altogether about 13 RNA molecules per nucleus. Insertion transcripts large enough to be potential precursors to 28S rRNA represent less than one molecule per nucleus. It was shown by sandwich hybridization that at least some of the insertion transcripts are derived from rDNA. No significant difference was found between insertion transcripts in RNA extracted from ovaries, embryos, larvae, pupae or adult flies. Unless a mechanism other than splicing is involved, ribosomal genes with insertions cannot contribute significantly to the synthesis of 28S rRNA. A cytoplasmic RNA ~1 kb long, which is complementary to a short insertion and to ribosomal gene sequences flanking both sides of the insertion, was found. The abundance of this short unspliced RNA is about 50 molecules per embryo cell.

Introduction

In the fruit fly (Drosophila melanogaster) there are about 150 tandemly repeated genes coding for 18S and 28S rRNA (ribosomal genes) on each nucleolus organizer. There is one nucleolus organizer on the X chromosome and one on the Y chromosome. Some ribosomal gen s are interrupted in the sequence coding for 28S rRNA by oth r s qu nc s called ribosomal insertions (Glover and Hogness, 1977; Wellauer and Dawid, 1977; White and Hogness, 1977; Pellegrini, Manning and Davidson, 1977). Ribosomal DNA insertions vary in siz and s qu nc but occur at th sam position in the 28S rRNA coding sequence. They can be divided into two groups, called type 1 and type 2, which are not homologous (Wellauer and Dawid, 1978; Dawid, Wellauer and Long, 1978; Wellauer, Dawid and Tartof, 1978). Type 1 insertions occur in 50% of the ribosomal genes on the X chromosome only, while type 2 insertions are present in about 15% of the ribosomal genes on the X and the Y chromosome. Type 1 insertions occur in size classes ranging from 0.5 kb to more than 5 kb. The short insertions are homologous to the right end of long insertions. Sequences homologous to type 1 insertions have been found outside the rDNA locus in the genome of D. melanogaster (Dawid and Botchan, 1977).

It has now been demonstrated for a number of viral and eucaryotic genes with intervening sequences that a precursor RNA complementary to the chromosomal gene sequence is made. The intervening sequences are later removed from the RNA by a splicing mechanism (see reviews by Crick, 1979 and Dawid and Wahli, 1979). If transcription of D. melanogaster ribosomal genes with insertions occurred by the same mechanism, there should be transcripts in nuclear RNA that contain insertion sequences. To test this possibility, we have analyzed the transcription of type 1 insertion sequences using three different technical approaches.

Results

RNA in D. melanogaster Embryos

D. melanogaster (Oregon R) embryo RNA was extracted from crude nuclear pellets, cytoplasmic fractions and whole embryos as described in Experimental Procedures. DNA was removed by several cycles of digestion with DNAase I. Integrity of the RNA was routinely tested by electrophoresis in agarose gels run in the presence of 5M urea. Figure 1 shows the various RNA preparations used in this study after gel electrophoresis. In cytoplasmic RNA, three prominent bands are seen corresponding to 18S rRNA and the two components of 28S rRNA which separate upon denaturation because of a hidden break introduced during processing (Jordan, 1975; Wellauer and Dawid, 1977). Larger discrete bands corresponding to the rRNA precursor and its processing products appear in nuclear RNA. Mature rRNA present in the nuclear fraction is partly due to cytoplasmic contamination.

The quantity of RNA and DNA in each cell fraction was determined. Since embryo cells are diploid, we were able to calculate the amount of RNA per cell for the crude nuclear, cytoplasmic and total homogenate fractions. The results are listed in Table 1. The value of 0.2 pg RNA per cell in the nuclear pellet includes contaminating cytoplasmic RNA. The degree of cytoplasmic contamination will not aff ct r sults exper-

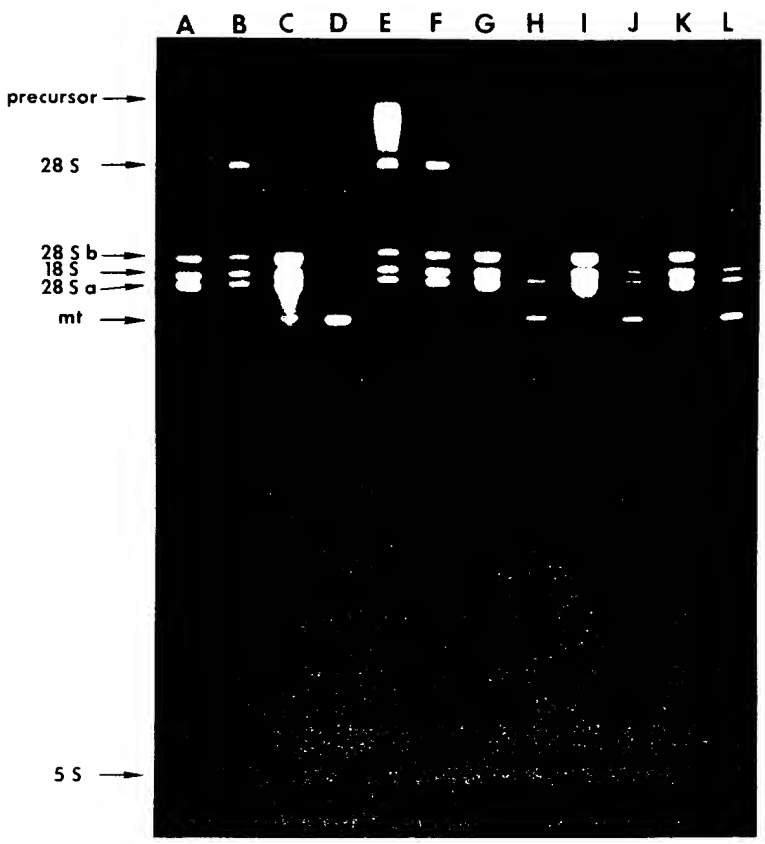

Figure 1. Gel Electrophoresis of D. melanogaster RNA

RNA samples were prepared and electrophoresed in a horizontal 0.8% agarose gel in the presence of 5 M urea, as described in Experimental Procedures, and stained with ethidium bromide. The prominent rRNA bands are indicated. The large mitochondrial rRNA (mt) has a very low G+C content (Klukas and Dawid, 1976) and is retained by the oligo(dT) column. The RNA samples are as follows. Ovary cytoplasmic (A); ovary nuclear (B); embryo cytoplasmic (C); embryo cytoplasmic poly(A)$^+$ after two passages through oligo(dT) (D); embryo nuclear (E and F) [(E) shows RNA prior to DNAase I digestion, while (F) shows RNA at the final stage of preparation]; larvae total (G); larvae total poly(A)$^+$ (H); pupae total (I); pupae total poly(A)$^+$ (J); adult flies total (K); adult flies total poly(A)$^+$ (L). Poly(A)$^+$ RNA samples H, J and L were run over only one oligo(dT) column.

essed in number of molecules per nucleus because the experiments described below were done with the same RNA preparations used for the RNA and DNA determinations shown in Table 1. A more detailed quantitation of various RNA species in these cell fractions will be considered below.

RNA Molecules Containing Ribosomal Insertion Sequences

A map of a ribosomal gene and of type 1 insertions is shown in Figure 2, together with a description of all the DNA fragments used in this study. We shall continue to refer to these DNA fragments by number throughout the paper.

We looked for RNA complementary to type 1 ribosomal insertions using the technique developed by Alwine, Kemp and Stark (1977). RNA samples were electrophoresed in agarose gels after denaturation with glyoxal (McMaster and Carmichael, 1977). They were transferred to diazobenzyloxymethyl paper (DBM paper) to which they become covalently bound. When filter-bound embryo nuclear RNA was hybridized with the cloned gene fragment 1, the same prominent RNA bands appeared as in a gel stained with ethidium bromide (Figure 3). The largest RNA band is the known rRNA precursor of 8.0 kb, measured previously by electron microscopy (Dawid et al., 1978). This experiment showed that no abundant rRNA transcripts much larger than 8 kb exist in embryo nuclei. Minor bands between 8 and 4 kb are probably intermediates in the processing of rRNA, and will be described elsewhere.

Cytoplasmic and nuclear RNA transferred to DBM paper were hybridized with cloned insertion sequences. The Sma I fragment [9], present only in large insertions, and the Bam HI fragment [11], derived from the right end of a large insertion and complementary to small insertions, were used separately. The resulting autoradiographs are shown in Figure 4. The Sma I fragment hybridized to some nuclear RNA molecules between 5 and 10 kb in size (lane B). The Bam HI fragment hybridized to more discrete and more prominant nuclear RNA bands (lanes D and E). Furthermore, the latter fragment hybridized to a cytoplasmic RNA molecule of about 1 kb (lane C). The size of RNA bands was determined by rehybridizing the same filters with a cloned continuous rDNA repeat and using the known size of rRNA molecules as internal standards.

Since the insertion probes were subcloned and thus completely free of ribosomal sequences, v ry low backgrounds obtained after hybridization and long exposures of the autoradiographs were possible. W estimated that the nuclear RNA bands shown in Figur 4 represent between <1 and 10 copi s per c ll, depending on the intensity of the band. Such quanti-

Table 1. Amount of RNA in D. melanogaster Embryos

Cell Fraction	% Total RNA	$\frac{RNA}{DNA}$ by Mass	RNA per Cell (pg)
Total Homogenate	100	9.60 ± 0.87 (5)	2.7
Nuclear Pellet	7.5 (6)	0.72 ± 0.17 (6)	0.2
Cytoplasmic Supernatant	93 (1)		2.5

RNA and DNA were quantitated in each cell fraction as described in Experimental Procedures. The results were expressed in amount per cell by assuming that each embryo nucleus contains 0.28 pg of DNA (Fristrom and Yund, 1973). The number of different RNA preparations tested is indicated in parentheses.

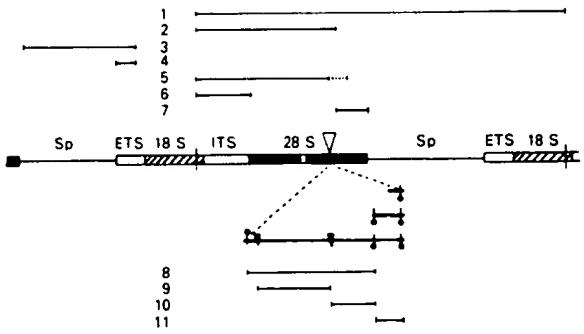

Figure 2. DNA Fragments Used in This Study

The map of a single continuous rDNA repeat is shown. The gene is separated from the neighboring genes by nontranscribed spacer (Sp). The external and internal transcribed spacers (ETS and ITS) and the 18S and 28S rRNA coding regions are indicated. The single site recognized by the restriction endonuclease Eco RI is indicated by a vertical bar (|). The point in the 28S rRNA coding sequence where insertions occur is indicated by a triangle (▽). Under the gene map are maps of the most frequent type 1 insertions of 0.5 kb, 1 kb and 5 kb. The sites recognized by the following restriction endonucleases are shown: Hind III ( ); Sma I (▼); Bam HI ( ). Fragments derived from the gene are shown above the gene map and are numbered as follows. Cloned Eco RI fragment from the continuous rDNA repeat DmrY22 [1]; Eco RI-Hind III fragment from the structural part of the gene [2]; Hind III-Hae III fragment from the spacer [3]; Taq I-Hae III fragment from the ETS [4]; Eco RI fragment from the cloned "half repeat" with type 2 insertion Dmra54 [5] [(– – –) type 2 insertion sequence]; Eco RI-Hae III fragment derived from Dmra54 [6]; Hind III gene fragment derived from the cloned "half repeat" with type 2 insertion Dmrb51 [7]. Details about the cloned rDNA repeats used here (DmrY22, Dmra54 and Dmrb51) have been published by Dawid et al. (1978). The mapping of some of these restriction sites will be described elsewhere. Fragments derived from type 1 insertions are shown below the map of insertions and are numbered as follows. Hind III-Bam HI fragment cloned in Hind III-Bam HI double-digested pBR322 [8]; Sma I fragment cloned in pMB9 using the isoschizomer Xma I to produce 5′ overhanging ends [9]; Sma I-Bam HI fragment [10]; Bam HI fragment cloned in pBR322 [11].

tative estimates were made by including diluted insertion DNA fragments as markers on parallel lanes in the gels, and by comparison with test experiments with diluted rRNA and rDNA fragments hybridized to rDNA. Because of the variability between different experiments (compare lanes D and E in Figure 4) and because of a number of assumptions made in the calculations, these numbers should be taken as a rough estimate. Nevertheless, it is clear that insertion transcripts exist but are quite rare compared to the mass of rRNA made in embryo cells. Better quantitative determinations of insertion transcripts were obtained by liquid hybridizations, as described below.

Several experiments with poly(A)$^+$ RNA fractions failed to show any hybridization with insertion DNA probes. We concluded that none of the observed insertion transcripts contains poly(A) long enough to bind to oligo(dT)-cellulose.

Quantitation of Insertion Transcripts by Kinetics of Hybridization

To quantify insertion transcripts, we hybridized in liquid excess unlabeled RNA with insertion DNA fragments labeled to a high specific activity. Hybrids were assayed by chromatography on hydroxyapatite. Some precautions had to be taken to obtain reliable data, as described in Experimental Procedures.

Embryo nuclear RNA was hybridized to the insertion fragments Sma I, Sma I-Bam HI and Bam HI (insertion fragments 9, 10 and 11, respectively). For a quantitative comparison, embryo nuclear RNA was also hybridized to a fragment derived from the structural part of the gene [2], and to a fragment derived from the external transcribed spacer [4], homologous to all rRNA precursors, nascent as well as complete. Results are shown in Figure 5A and the concentrations of the various transcripts are given in Table 2. The more abundant insertion transcripts, those homologous to th Bam HI insertion fragment [11], represent about 13 molecul s per nucleus. The transition in the Rot curve appears complex, suggesting that different RNA molecul s occur which are homologous to either part or all of the Bam HI insertion fragment. Transcripts homologous to other r gions of the 5 kb ins r-tion represent less than one molecule per nucleus, more than three orders of magnitude below the concentration of rRNA precursors, which is 4700 copies per nucleus. There are about 1300 mature precursor rRNA molecules per nucleus, leaving an estimated 3400 nascent rRNA chains (Table 2).

Embryo cytoplasmic RNA was hybridized to the Bam HI insertion fragment [11] and to a fragment derived from the structural part of the gene [2]. Results are shown in Figure 5B and Table 2. The cytoplasmic insertion transcript represents about 50 molecules per cell. The saturation level of the Rot curve indicates that the transcript is homologous to only a part of the Bam HI insertion fragment.

The relevant numbers from these experiments are the ratios between insertion transcripts and rRNA precursor molecules. These ratios are independent of possible errors in the determination of RNA, DNA and genome size. Furthermore, all liquid hybridizations were performed with the same nuclear or cytoplasmic RNA preparation.

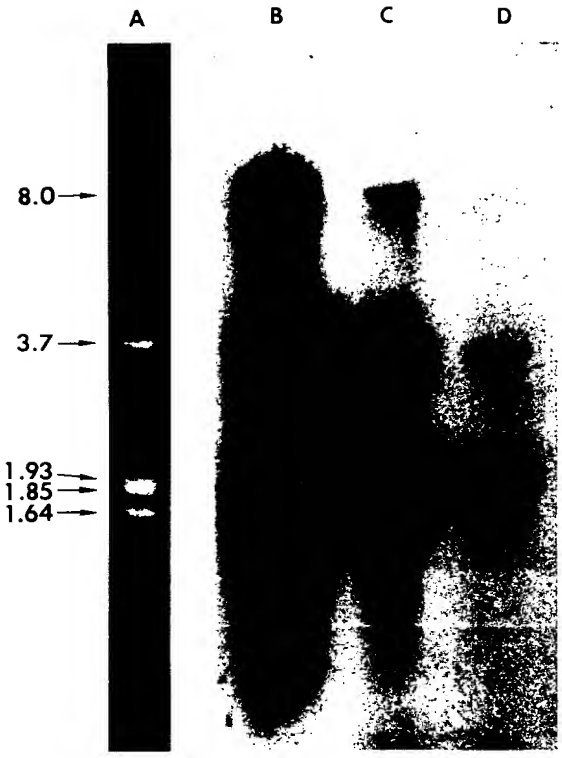

Figure 3. RNA Homologous to Ribosomal Gene Sequences

Embryo nuclear RNA was glyoxalated; 3.9 μg were electrophoresed in a horizontal 0.8% agarose gel and stained with ethidium bromide (A). 10 μg (B), 1 μg (C) and 0.1 μg (D) were electrophoresed in another 0.8% agarose gel, transferred to DBM paper and hybridized for 2 days with ^{32}P-labeled pDmrY22 (5×10^6 cpm/ml at 4×10^7 cpm/μg), which is the cloned gene fragment 1. The sizes of the rRNA precursor, 28S, 28Sb, 18S and 28Sa molecules are given in kb as determined by electron microscopy (Dawid et al., 1978).

Identification of RNA Molecules Containing Ribosomal and Insertion Sequences

We have so far established that, although rare, insertion transcripts exist in D. melanogaster embryos. Since sequences homologous to ribosomal insertions exist outside the rDNA locus in the genome of D. melanogaster (Dawid and Botchan, 1977), it remains to be established what sequences flank the insertions in those transcripts. We tested whether insertion transcripts were derived from ribosomal DNA by "sandwich-hybridization," a method first described by Dunn and Hassell (1977).

In the first series of experiments, we transferred restriction fragments of insertion DNA onto nitrocellulose filters. These filters were hybridized with unlabeled nuclear or cytoplasmic RNA and washed extensively to remove unhybridized RNA. They were then hybridized again with ^{32}P-labeled DNA fragments derived from the ribosomal gene. These DNA probes did not share any homology with insertion DNA fragments on the filters because they were derived from a continuous repeat of cloned rDNA. Hybridization could therefore only occur with tails of RNA molecules that had hybridized to an insertion fragment during the first round of hybridization. The results showed that rRNA molecules which contain insertion sequences exist (Figure 6). In nuclear RNA most of these molecules are complementary to the Bam HI insertion fragment [11], a few to the Sma I fragment [9], but none could be detected that were hybridized to the Sma I-Bam HI fragment [10]. DNA probes derived from gene sequences either to the left or to the right of the point of insertion were used separately, and showed that insertion transcripts complementary to the Bam HI fragment are complementary to gene sequences from either side of the insertion. Since a number of different insertion transcripts exist in nuclear RNA, however (see Figure 4), this experiment does not answer whether any *one* molecule contains ribosomal gene sequences flanking both sides of the insertion.

We also found cytoplasmic RNA complementary to the Bam HI insertion fragment and to ribosomal gene sequences (Figure 6, lanes G, H and I), which is almost certainly the 1 kb insertion transcript described above because it is the only detectable cytoplasmic insertion transcript (Figure 4). The 1 kb RNA hybridized to gene sequences flanking both sides of the insertion. In this case it is probable that a single cytoplasmic RNA species consists of sequences that bridge the insertion. Since it is 1 kb long, it can contain only a short insertion sequence (perhaps 0.5 kb) and only part of the rRNA sequences. It is worth pointing out that the DNA probes used for gene sequences left or right of the insertion were derived from separate clones ("half-repeat clones"; see Dawid et al., 1978). Consequently, such fragments cannot be contaminated by 28S rRNA coding sequences from the other side of the insertion.

We did a second set of experiments to answer a different question. Are there rRNA precursor molecules that contain insertion sequences? We knew from the results shown in Figure 4 that insertion transcripts large enough to be potential rRNA precursors are very rare. A DNA fragment corresponding to most of the rDNA spacer, including the external transcribed spacer [3], was transferred to a nitrocellulose filter and hybridized with embryo nuclear RNA. Only rRNA precursors, nascent as well as full-length, can hybridize, since no structural gene sequence is present in this spacer DNA fragment. The filters were then hybridized with cloned insertion sequences. Very high sensitivity was achieved in this experiment because of the low background obtained with the pure insertion probe. The results are shown in Figure 7. By comparison with the experiment run in parallel, where 1000 fold diluted precursor rRNA molecules were hybridized with a fragment derived from th structural part of the gene [6], we concluded that rRNA precursor molecules containing insertion sequences are present in less than one copy per embryo nucleus. Even

though a faint positive results was obtained, this experiment does not prove the existence of such molecules, because the size and nature of the RNA which hybridizes to both sequences (spacer and insertion) is not known. The reason for this uncertainty is the fact that rearrangements occur within D. melanogaster rDNA (Glover, 1977; Dawid et al., 1978). A rare RNA molecule containing both spacer and insertion sequences could be derived from an rDNA region of atypical structure.

Insertion Transcripts in Different Stages of Development

All the experiments described above were done with RNA extracted from 3–18 hr old embryos. We prepared RNA from other stages of development in order to answer the following questions. Are insertion transcripts more abundant in certain stages? Are the insertion transcripts found in embryos, in particular the 1 kb cytoplasmic RNA, also present in other stages? We tested these possibilities by transferring RNA from agarose gels to DBM paper and by hybridizing the filters with cloned insertion sequences, as was done with embryo RNA.

In a Drosophila ovary, 15 nurse cells actively synthesize rRNA which is accumulated into the single oocyte (Mermod, Jacobs-Lorena and Crippa, 1977). Whole ovaries were manually dissected from D. melanogaster females. Nuclear, cytoplasmic and total RNA was extracted. As seen in Figure 1, nearly intact RNA was obtained. After transfer to DBM paper and hybridization with labeled insertion DNA, no major difference was found between insertion transcripts in ovaries and embryos (Figure 8). In an experiment with good resolution among large RNA molecules and very high sensitivity, a weak band of about 12 kb hybridized to the insertion probe, both in ovary and embryo nuclear RNA. The RNA in this band is certainly present in <11 copy per 10 cells in embryo nuclei, based on a comparison with the autoradiographic signal of all the other insertion transcripts, whose total concentration is given in Table 2.

Because of high levels of endogenous RNAase activity, we could not prepare intact cytoplasmic and nuclear RNA from larvae, pupae or adult flies. However, we developed a technique which allowed us to prepare nearly intact total RNA from these stages, as shown in Figure 1. After transfer to DBM paper and hybridization with the Bam HI insertion fragment [11], we concluded that no major difference exists b tw n ins rtion transcripts in all stages examined (Figure 9). Th same major insertion transcripts as in embryos wer found in all stag s tested. The relative abundance of insertion transcripts is lower in later stages of d velopm nt than in embryos. The 1 kb insertion transcript found in th cytoplasm of embryos is also pr s nt in ovari s, larva , pupae and adult flies. No cl ar band appeared after hybridization of total RNA from embryos, larvae, pupae and adult flies with a DNA fragment derived from a large insertion [8], and none of the detected insertion transcripts was present in poly(A)$^+$ RNA (data not shown).

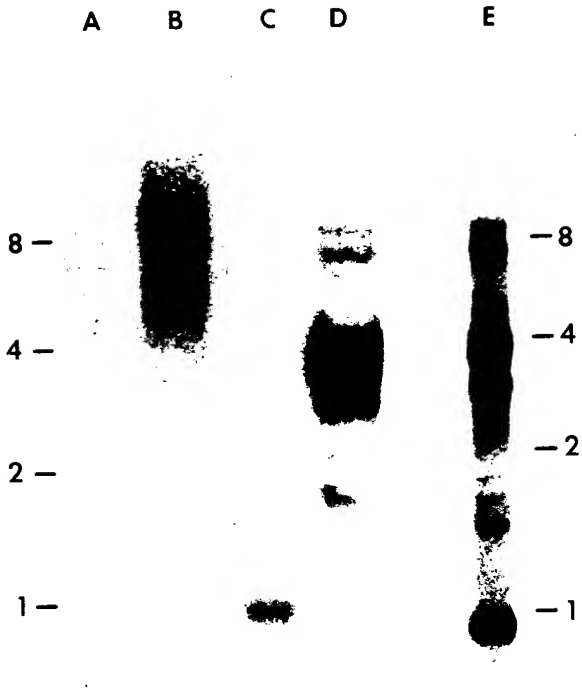

Figure 4. RNA Homologous to Ribosomal Insertions

Cytoplasmic and nuclear RNA from D. melanogaster embryos were glyoxalated, electrophoresed in a vertical 0.8% agarose gel and transferred to DBM paper. Lanes A–D represent a single gel, while lane E is a separate experiment. Lanes A and C were loaded with 50 μg cytoplasmic RNA, lanes B, D and E with 30 μg nuclear RNA. Lanes A and B were hybridized for 2 days with 2×10^6 cpm/ml of ^{32}P-labeled fragment Sma I [9] (8×10^7 cpm/μg) which is complementary to a part of large insertions, lanes C and D for 2 days with 2×10^6 cpm/ml of ^{32}P-labeled fragment Bam HI [11] (3×10^7 cpm/μg) which is complementary to small insertions and to the right end of large insertions, lane E for 5 days with 1×10^6 cpm/ml of ^{32}P-labeled fragment Bam HI [11] (8.2×10^7 cpm/μg). Sizes are given in kb.

Discussion

Synthesis of rRNA in D. melanogaster Embryos

Transcription of rRNA genes in D. melanogaster embryos starts at the early cellular blastoderm stage (McKnight and Miller, 1976), which occurs 2.5–3 hr after oviposition. We have prepared RNA from 3–18 hr old embryos of D. m lanogaster and determined by quantitation of gel patterns that there are about 1300 full-l ngth rRNA precursor mol cul s per average embryo nucleus. From the kinetics of hybridization of nuclear RNA to a DNA fragment derived from the external transcribed spacer, we determined that the total number of rRNA pr cursor mol cules, nascent

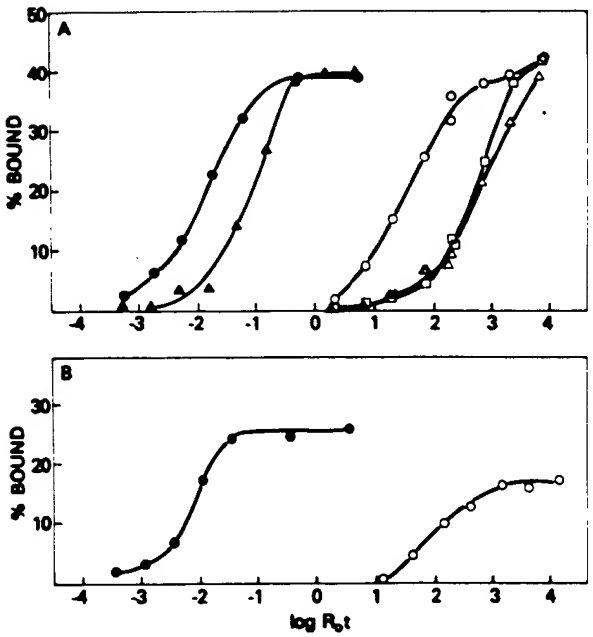

Figure 5. Quantitation of Insertion Transcripts

Nuclear and cytoplasmic RNA from embryos were hybridized in liquid to ^{32}P-labeled DNA fragments. Hybrids were assayed on hydroxyapatite. Results are expressed in percentage of the DNA probe bound to hydroxyapatite. For asymmetric transcription, the maximum theoretical level is 50%. (A) nuclear RNA hybridized to the ribosomal gene fragment 2 (-●-), to the external transcribed spacer fragment 4 (-▲-), to the Bam HI insertion fragment [11] (-○-), to the Sma I insertion fragment [9] (-△-) and to the Sma I-Bam HI insertion fragment [10] (-□-). The $Rot_{1/2}$ values for the three curves with insertion DNA probes were determined assuming a saturation plateau at 43%. The ratio nuclear RNA/probe was such that RNA molecules present in a single copy per cell were in 10 fold excess. (B) cytoplasmic RNA hybridized to the ribosomal gene fragment 2 (-●-) and to the Bam HI insertion fragment [11] (-○-). Only 33% of gene fragment 2 is complementary to cytoplasmic rRNA (see Figure 2). The ratio of cytoplasmic RNA to probe was such that RNA molecules present in 10 copies per cell were in 15 fold excess.

as well as complete, is 4700 per average embryo nucleus (Figure 5 and Table 2). McKnight and Miller (1976) examined spread nucleolar chromatin from D. melanogaster embyros at the cellular blastoderm stage and estimated that about 4000 nascent rRNA chains are synthesized per nucleolus organizer. These values are in quite good agreement, but should still be taken as a rough estimate. The arguments presented below will rest on more solid relative numbers of RNA molecules measured under constant conditions.

Ribosomal Ins rtion Transcripts Ar Rar in Nuclear RNA

Approximately half of the ribosomal genes on the X chromosome are interrupted by an insertion of type 1 (Wellauer et al., 1978). If interrupted ribosomal genes produced rRNA via a splicing mechanism in a manner analogous to other genes studied thus far, transcripts containing insertion sequences would be quite abundant in nuclear RNA. This prediction is true no matter how quickly splicing occurs, because transcription must proceed across the entire insertion sequence before splicing can take place. This prediction assumes that the insertion would be transcribed at the same rate as the gene regions and that splicing would commence only after the entire insertion has been transcribed. Alternate hypotheses are discussed below. Another prediction is that ribosomal insertion transcripts up to 12 kb long would exist, provided their processing rate is similar to that of normal 8 kb rRNA precursor.

We detected insertion transcripts in nuclear RNA (Figure 4). With a DNA probe specific for large insertions, we found transcripts ranging from 5–10 kb, not discrete in size. Quantitation of these transcripts by kinetics of hybridization showed that they represent only about one copy per nucleus (Figure 5, Table 2). With another DNA probe complementary to the right end of the 5 kb insertion and to short insertions, we detected more discrete RNA bands, from 1–8.5 kb (Figure 4), which represent altogether about 13 copi s per nucleus (Figure 5, Table 2). Since sequenc s homologous to rDNA insertions occur in the D. melanogaster genome outside the nucleolus organiz r (Dawid and Botchan, 1977), we had to establish whether any of the detected insertion transcripts were derived from rDNA. Sandwich hybridizations allowed us to determine that at least some, and probably a majority, of the insertion transcripts also carry ribosomal sequences (Figure 6). The total number of insertion transcripts is very low, however, compared to the 3000–4000 *nascent* ribosomal transcripts in an average embryo nucleus.

A single ribosomal gene with a 5 kb insertion transcribed at the rate known for rDNA would carry approximately 80 RNA polymerase molecules over a 5 kb insertion and would therefore produce about 80 nascent RNA chains containing insertion sequences. This estimate derives from the packing density of polymerase molecules on ribosomal transcription units (McKnight and Miller, 1976). We observed many fewer RNA molecules homologous to the left end of 5 kb insertions, indicating that not even a single rRNA gene with a 5 kb insertion is transcribed at a rate similar to that of continuous rDNA units. Each nucleolus organizer on an X chromosome contains about 50 rRNA genes with a 5 kb insertion. The rare ribosomal insertion transcripts complementary to the left part of large insertions are likely to result from occasional abortive transcription into the insertion sequence. This interpretation is supported by two observations: first, such RNA molecules are heterogeneous in size, showing a "smear" between 5 and 10 kb (Figur 4); and second, in sandwich hybridization, no rRNA could b detected that hybridized to an insertion fragment from

Table 2. Concentration of Sequences Complementary to rDNA Structural and Insertion Sequences in Embryo RNA

	Nuclear RNA		Cytoplasmic RNA	
DNA Probe	Rot$_{1/2}$	Molecules per Cell	Rot$_{1/2}$	Molecules per Cell
Gene Fragment 2	1.25×10^{-2}	36000[a,b]	7.5×10^{-3}	620000[a]
Gene Fragment 4	9.5×10^{-2}	4700[c]		
Insertion Fragment 9	6.6×10^{2}	0.68[c]		
Insertion Fragment 10	5.6×10^{2}	0.80[c]		
Insertion Fragment 11	3.5×10^{1}	13[c]	1×10^{2}	47[c]

[a] Calculated as this example:

$$8\text{ kb RNA molecules/nucleus} = \frac{\text{molecules/mol} \times \text{pg RNA/nucleus} \times 8\text{ kb nuclear RNA/total nuclear RNA}}{\text{pg of 8 kb RNA/mol}}$$

$$= \frac{6.02 \times 10^{23} \times 0.2 \times 0.03}{8 \times 10^{3} \times 348 \times 10^{12}} = 1297.$$

Other RNA molecules were calculated the same way, using appropriate numbers. The fraction represented by an RNA species in the RNA preparation was determined by densitometric tracing of RNA gels, as described in Experimental Procedures. The area under each RNA peak was measured and divided by the total area.

[b] Sum of the number of 28S rRNA molecules and of all 28S rRNA precursor molecules. The number of each individual RNA species (8 kb rRNA, 28S rRNA precursors and 28S rRNA) was determined according to the formula in footnote a. Nascent rRNA chains were not taken into consideration. According to our unpublished data, nascent rRNA molecules represent less than 10% of the total number of RNA molecules homologous to gene fragment 2 in nuclear RNA.

[c] The number of molecules homologous to gene fragment 2 was used as kinetic standard.

the central part of a large insertion (Figure 6).

There are about 20 ribosomal genes with short insertions in the nucleolus organizer of the X chromosome. We believe that full-length transcripts of such genes may exist, based on the following observations: an RNA band of 8.5 kb homologous to the sequence of a short insertion was found (Figure 4); insertion transcripts complementary to short insertions were found to hybridize to ribosomal gene sequences derived from either side of the insertion (Figure 6); individual RNA molecules complementary to the external transcribed spacer and to short insertion sequences were detected by sandwich hybridization (Figure 7). Thus initiation of transcription on a gene with a short (0.5 or 1 kb) insertion may occur and, once initiated, the transcript may proceed to the end of the 28S rRNA gene. It is clear that these potential rRNA precursors exist in less than one copy per nucleus (Figures 4 and 7). Even if these ribosomal insertion transcripts were spliced correctly, their contribution to the synthesis of 28S rRNA would be insignificant, because their concentration is more than three orders of magnitude below the concentration of the 8 kb rRNA precursor.

The above conclusions assume a splicing mechanism analogous to that seen with other genes (reviewed by Crick, 1979; Dawid and Wahli, 1979). Several mechanisms can be envisioned that would lead to significant use of interrupted rRNA g n s and still be compatible with our results. First, RNA polymeras could bypass the insertion and continue transcription at its distal end. Second, splicing might occur in multiple steps while the nascent RNA is still attached to the RNA polymerase. If this "stuttered" splicing proceeded as fast as polymerization in steps of 50–100 nucleotides, our results indicate that no more than one interrupted gene would be used. Third, RNA polymerase might traverse insertion sequences at a faster rate than gene sequences. If interrupted rRNA genes were transcribed as frequently as uninterrupted genes, the acceleration would have to be several hundred fold to account for our results. Such a transcriptional rate is almost certainly impossible.

The unprecedented mechanisms mentioned above that would allow utilization of interrupted rRNA genes at a high rate become less probable when we compare our results with those for other interrupted genes that are productively transcribed. The rDNA in some strains of Tetrahymena is interrupted (Wild and Gall, 1979) and must be used, since no uninterrupted gene exists in these strains. Din et al. (1979) have shown that a large precursor RNA including the intervening sequence is produced in these cells and can readily be detected. Even more striking is the comparison with the single-copy gene for ovalbumin. Roop et al. (1978) have shown that, after stimulation of chicken with estrogen, the nuclei from oviduct tubular gland cells contain at steady state about 250 transcripts complementary to ovalbumin gene intervening sequences. These comparisons support our suggestion that interrupted rRNA genes in D. melanogaster are not transcribed in a way that contributes effectively to rRNA production.

It is possible, however, that transcription on inter-

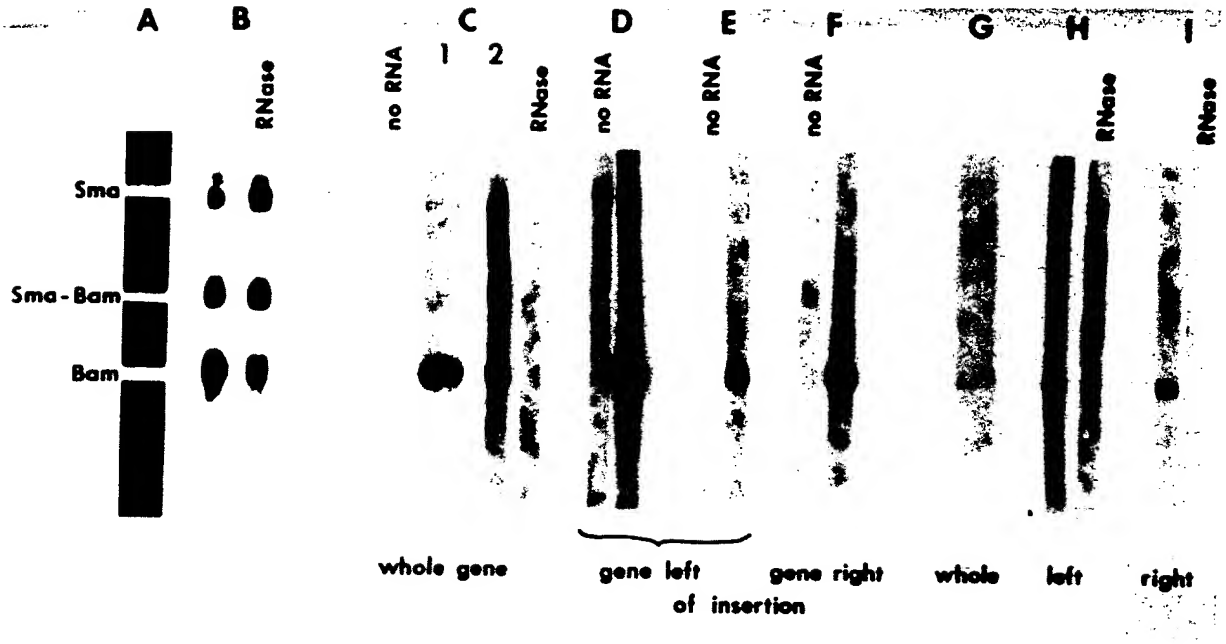

Figure 6. Insertion Transcripts Homologous to rDNA

The cloned insertion fragment Hind III-Bam HI [8] was digested with Sma I. The resulting digest was electrophoresed in a 0.8% agarose gel together with the cloned insertion fragment Bam HI [11]. DNA was stained with ethidium bromide (A) and transferred to a nitrocellulose filter. The filter was cut into strips. Two strips were hybridized with ^{32}P-labeled insertion fragments [8 and 11] to localize the DNA on the filter (B). The positions of insertion fragments Sma I [9], Sma I-Bam HI [10] and Bam HI [11] are indicated. The short fragment Hind III-Sma I was not retained quantitatively. Panels C, D, E and F are sandwich hybridizations with embryo nuclear RNA. Each filter was hybridized in 0.5 ml with 100 μg nuclear RNA, as described in Experimental Procedures, except C1, which was hybridized with 225 μg nuclear RNA. Panels G, H and I are sandwich hybridizations with embryo cytoplasmic RNA. Each filter was hybridized in 0.5 ml with 800 μg cytoplasmic RNA. The filters were then washed extensively. The ^{32}P-labeled DNA probes used in the second round of hybridization are indicated below the panels. Gene fragment 1 (whole gene), 1×10^6 cpm/ml at 8×10^7 cpm/μg (C1 and G) and 2×10^6 cpm/ml at 3.3×10^7 cpm/μg (C2). Gene sequence to the left of the insertion [5], 2×10^6 cpm/ml at 2×10^8 cpm/μg (D and H). Gene sequence to the far left of the insertion [6], 2×10^6 cpm/ml at 4.6×10^7 cpm/μg (E). Gene sequence to the right of the insertion [7], 2×10^6 cpm/ml at 2×10^8 cpm/μg (F and I). Control filters that were not hybridized with RNA but otherwise carried through all steps are indicated by "no RNA." Control filters treated with RNAase after the first hybridization, as described in Experimental Procedures, are indicated by "RNAase."

rupted rRNA genes initiates but that a strong stop signal prevents continuation of transcription into the insertion. The resulting abortive rRNA precursor would be 6.8 kb long, and we have not detected the accumulation of such molecules in Drosophila embryos (data not shown). While this result does not exclude such a mechanism, we hold it to be most probable that interrupted genes are in general not transcribed at all. This interpretation is consistent with the observation of McKnight and Miller (1976) that nucleolar chromatin from D. melanogaster embryos contains long stretches of silent DNA interspersed with active transcription units.

An Unsplic d Ribosomal Ins rtion Transcript in Cytoplasmic RNA

We have found a cytoplasmic RNA molecule, slightly less than 1 kb long, complementary to the sequence of a short insertion (Figure 4) and to ribosomal gene sequences from both sides of the insertion (Figure 6). This unspliced RNA is present in about 50 copies per cell (Figure 5). We do not know how it is transcribed or whether it has any function. It may be derived from a ribosomal gene with a short insertion by processing at unusual sites. There are alternatives to this simple explanation. Transcription could initiate within the sequence of the 28S rRNA gene upstream from th insertion. The 1 kb RNA also might be transcribed from rearranged rDNA sequences with unusual structure. Rearrangements in D. melanogaster rDNA are known to occur (Glover, 1977; Dawid et al., 1978).

The finding of an unspliced cytoplasmic rRNA molecule raises the possibility that D. melanogaster lacks a functional splicing machinery for rRNA, or at l ast that wild-type animals do not express such a mechanism. Two interpretations come to mind. Th first suggests that evolutionary ancestors of Drosophila

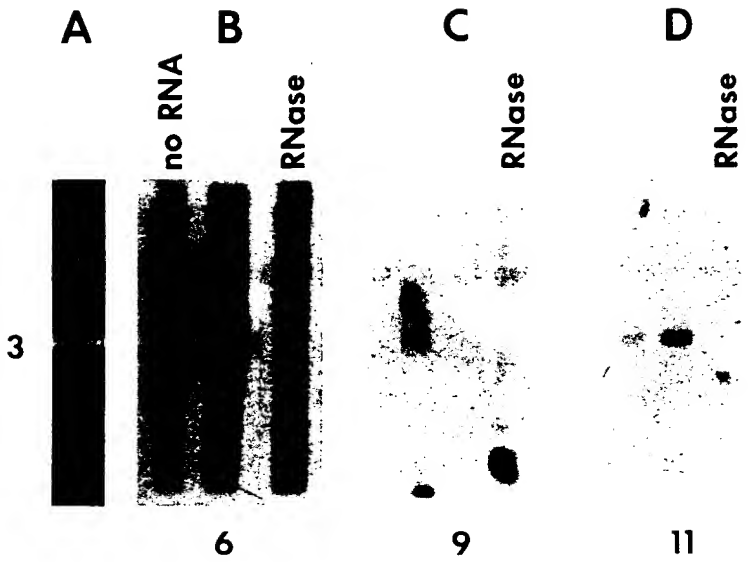

Figure 7. RNA Molecules Homologous to rDNA Spacer and Insertion Sequences

The rDNA fragment 3 (spacer fragment) was electrophoresed in a 0.8% agarose gel and stained with ethidium bromide (A). It was transferred to a nitrocellulose filter which was then cut into strips. Each strip was hybridized in 1 ml with embryo nuclear RNA, either 0.1 μg (B) or 100 μg (C and D). The ^{32}P-labeled DNA probes used in the second round of hybridization (2×10^6 cpm/ml) are indicated below the panels. Gene fragment [6], 4.6×10^7 cpm/μg (B); insertion fragment Sma I [9], 2×10^7 cpm/μg (C); insertion fragment Bam HI [11], 3.8×10^7 cpm/μg (D). A control filter that was not hybridized with RNA but otherwise carried through all steps is indicated by "no RNA." Control filters treated with RNAase after the first hybridization are indicated by "RNAase." All filters were autoradiographed on the same X-ray film.

ribosomal genes were interrupted, but that after evolution of genes without insertions the mechanisms necessary for splicing were lost. Some number of interrupted ribosomal genes was maintained, even though no longer functional, in the Drosophila genome. The second interpretation is that interrupted ribosomal genes were derived from continuous genes by an insertion event and that rRNA splicing functions never evoled in Drosophila. Transposable genetic elements are known to exist in Drosophila (Potter et al., 1979; Strobel, Dunsmuir and Rubin, 1979).

Ribosomal Insertion Transcripts in Different Developmental Stages

The major conclusion from this work—namely, that ribosomal genes with insertions cannot contribute significantly to the synthesis of 28S rRNA in D. melanogaster—has been elaborated in most detail for embryos but is also true for other stages of development. We have compared nuclear RNA from ovaries and embryos and found no significant difference (Figure 8). In total RNA from pupae, larvae and adult flies, we detected the same major insertion transcripts as in embryos—namely, a 4 kb molecule (the prominent nuclear insertion transcript in embryos) and a 1 kb molecule (known to be cytoplasmic in embryos)—although their concentrations in later stages were lower than in embryos (Figure 9). It remains possible that active transcription of ribosomal genes with insertions occurs in c rtain specialized c lls of D. melanogaster, but thes cells could not constitute a large part of the total mass of the animal at any of the stages of d v lopment that we tested. We can conclud that, in gen ral, xpr ssion of interrupt d ribosomal genes in wild-type D. melanogast r diff rs from the expression of other ucaryotic g nes with int rvening sequences.

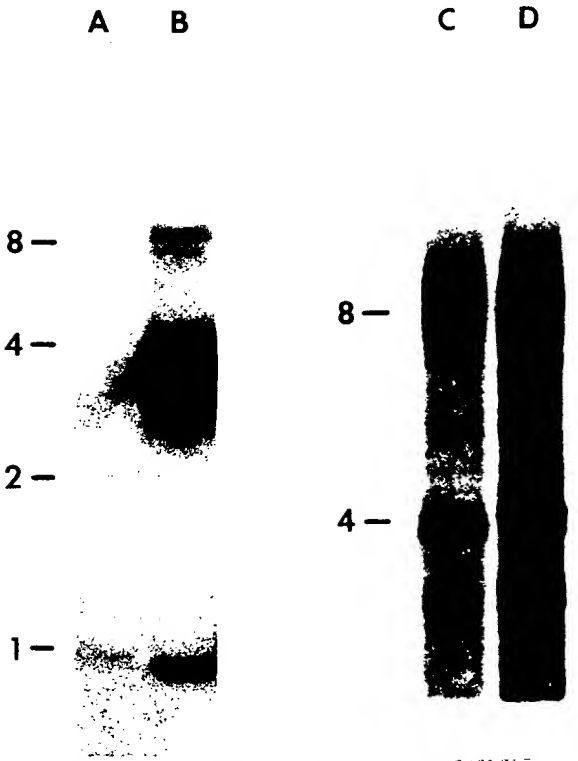

Figure 8. Insertion Transcripts in Ovaries and Embryos

Total RNA from ovaries and nuclear RNA from ovaries and embryos were glyoxalated, electrophoresed in a 0.8% vertical agarose gel and transferred to DBM paper. 1 pg of the insertion fragment Bam HI [11] mixed with 10 μg E. coli tRNA (A) and 100 μg of ovary total RNA (B) were run in parallel and hybridized for 5 days with 4×10^6 cpm/ml of 8.2×10^7 cpm/μg ^{32}P-labeled insertion fragment Bam HI [11]. 35 μg nuclear RNA from ovaries (C) or embryos (D) were run in parallel and hybridized for 6 days with a mixture of ^{32}P-labeled insertion fragments Hind III-Bam HI [8] (1.33×10^6 cpm/ml, 8.3×10^7 cpm/μg) and Bam HI [11] (0.66×10^6 cpm/ml, 4×10^7 cpm/μg). These two fragments represent almost the entire 5 kb insertion. Sizes are given in kb.

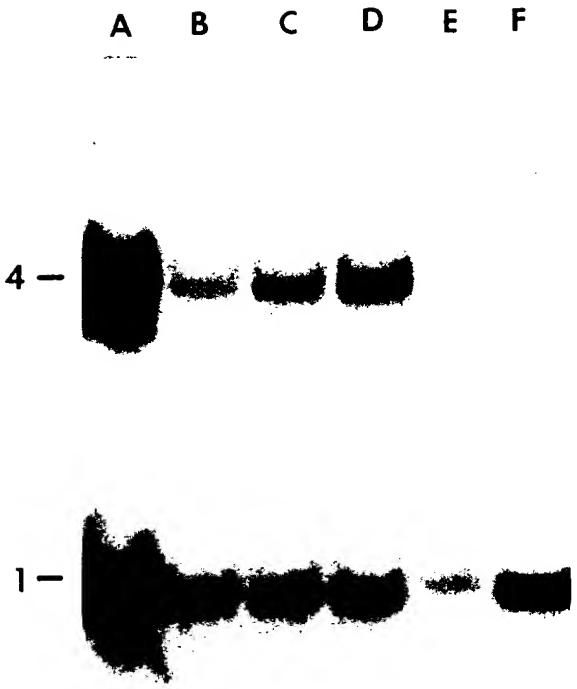

Figure 9. Insertion Transcripts in Different Developmental Stages

Total RNA (100 μg each) from embryos (A), larvae (B), pupae (C) and adult flies (D) were glyoxalated, electrophoresed in a vertical 0.8% agarose gel and transferred to DBM paper. 3 pg (E) and 10 pg (F) of the insertion fragment Bam HI [11] mixed with 10 μg E. coli tRNA were run in parallel lanes. The filter was hybridized for 5 days with ^{32}P-labeled insertion fragment Bam HI [11] (4×10^5 cpm/ml, 8.2×10^7 cpm/μg). Sizes are given in kb.

Experimental Procedures

Rearing and Harvest of Organisms

D. melanogaster Oregon R strain P2 (obtained from K. Tartof) were reared at 25°C in population cages according to the method of Travaglini and Tartof (1972). Ovaries were manually dissected from chilled females in isotonic buffer [30 mM Tris-HCl (pH 8.3), 100 mM NaCl, 10 mM $CaCl_2$]. 3–18 hr old embryos were collected by washing the yeast paste fed to the flies through mesh screens. They were rinsed with 70% ethanol, dechorionated in 50% Chlorox for 2 min, further rinsed in 70% ethanol and decanted twice in a large volume of 0.9% NaCl, 0.01% Nonidet P40. Larvae were collected at the third instar stage.

Preparation of RNA

Total RNA from dechorionated embryos, larvae, pupae and adult flies was prepared as follows. The organisms were chilled in a Sorvall Omni-Mix. About 25 ml of a 1:1 mixture of SDS buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM EDTA, 0.5% SDS] and phenol-chloroform (distilled phenol:chloroform:isoamyl alcohol 100:99:1) were added per g of organism and immediately blended for 3 times 10 sec at full speed. The organic phase was removed and two extractions with chloroform containing 1% isoamyl alcohol were performed. The cycle of one phenol-chloroform and two chloroform extractions was repeated twice, according to the method of Spradling, Hui and Penman (1975). The aqueous phase was adjusted to 0.3 M NaCl and precipitated with ethanol. After a second precipitation with ethanol, the pellet was dissolved in H_2O at 0°C and adjusted to 10 mM Tris-HCl (pH 7.4) 0.5 M NaCl, 50 mM $MgCl_2$. Pancreatic DNAase I (Worthington), which had been passed over a column of agarose 5′-(p-aminophenyl phosphoryl) uridine 2′(3′) phosphate (Miles-Yeda) in 20 mM sodium acetate, (pH 5.0) to remove traces of RNAase, was added (5 μg per mg DNA in the sample) and incubated for 30 min at 37°C. The sample was adjusted to 0.5% SDS, 50 mM EDTA. Proteinase K (Boehringer-Mannheim) was added to 0.2 mg/ml and incubated for 30 min at 37°C. The sample was spun at 12,000 rpm for 30 min at room temperature in the JS-13 rotor of the Beckman J-21 centrifuge. The supernatant was extracted with two cycles of phenol-chloroform and chloroform extractions and precipitated with ethanol.

Cytoplasmic RNA from ovaries and embryos was prepared as follows. Ovaries and dechorionated embryos were homogenized in cold isotonic buffer (10 ml per g organism) containing 0.5% Nonidet P40 and 50 μg/ml polyvinyl sulfate. The homogenate was spun at 12,000 rpm for 10 min in the JS-13 rotor. The supernatant was adjusted to 0.5% SDS, 25 mM EDTA and extracted as described above for total RNA. Digestion with DNAase I and proteinase K and further extraction were as described above. Total RNA from ovaries was prepared as was cytoplasmic RNA, but without the 12,000 rpm spin.

Nuclear RNA from embryos was prepared as follows. Dechorionated embryos were homogenized as described for cytoplasmic RNA. The homogenate was filtered through a nylon bolting cloth (Nitex No. 44, Tetco), underlayered with a cushion of 1.5 M sucrose in isotonic buffer and spun for 12 min at 3000 rpm in the JS-13 rotor. The nuclear pellet was resuspended twice in isotonic buffer containing 0.5% Nonidet P40 and 50 μg/ml polyvinyl sulfate and spun for 5 min at 3000 rpm over the sucrose cushion. The final nuclear pellet was lysed at room temperature in SDS buffer (5 ml per g of embryos), blended for 5 sec in the Sorvall Omni-Mix with an equal volume of phenol-chloroform and extracted as described for total RNA. Nuclear RNA from ovaries was prepared as follows. Dissected ovaries were collected in isotonic buffer containing 0.5% Nonidet P40, 50 μg/ml polyvinyl sulfate, 0.5 mM spermidine and 0.15 mM spermine, homogenized in the same buffer and spun for 5 min at 3000 rpm in the JS-13 rotor. The pellet was washed in the above buffer and spun 5 min at 3000 rpm. The pellet was lysed in SDS buffer and extracted with phenol-chloroform and chloroform as described for total RNA. DNAase I and proteinase K digestions and the following extractions were as described above.

The RNA samples used in liquid hybridizations were purified further, as follows. The nuclear RNA sample was passed over a Sephadex-G50 column in SDS buffer to remove digested deoxyribonucleotides after the DNAase I and proteinase K digestions, and was then extracted as described. The sample was digested with DNAase I and precipitated with ethanol 3 more times. After the fourth DNAase I digestion, it was extracted with phenol-chloroform and precipitated with ethanol. Total RNA and cytoplasmic RNA samples were digested a second time with DNAase I. All samples were passed over a Chelex 100 column.

All RNA samples were stored in H_2O at −20°C or −70°C. Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972) after boiling the RNA for 1 min in 1% SDS.

Quantitative determinations of RNA and DNA in embryo total homogenate, cytoplasmic supernatant and nuclear pellet were done by the method of Schmidt and Thannhauser (1945) and by the diphenylamine assay (Burton, 1956).

Electrophoresis of RNA and Transfer to DBM Paper

We developed a convenient agarose-urea gel system for routinely checking RNA preparations. The buffer used was 89 mM Tris-borate and 2.5 mM EDTA at pH 8.3 (Peacock and Dingman, 1967). A 2X mixture of agarose and buffer was autoclaved, cooled to 60°C and mixed with an equal volume of 10 M urea. The solution was poured in the cold over the gel apparatus (0.5 × 13 × 20 cm). The slot-former was immersed in H_2O saturated with diethylpyrocarbonate before being inserted into the agarose, which was allowed to gel

overnight at 4°C. Electrophoresis was then carried out at room temperature. RNA samples (<1 μg per band) were loaded in ≅5 M urea with 5% sucrose, 0.05% bromophenol blue, 10 mM EDTA and 0.05% SDS. After electrophoresis at 150 V and 25 mA for about 5 hr, the gel was stained in a large volume of 0.1 M ammonium acetate, 1 μg/ml ethidium bromide with gentle rocking for 1 hr. As seen in Figure 1, this procedure gave excellent resolution and was sufficiently denaturing to dissociate cytoplasmic 28S rRNA into its two components. Pictures of the gels under ultraviolet illumination were traced by densitometry as described by Hewlett et al. (1977). A linear response for the amount of RNA was obtained below 1 μg of RNA per discrete band.

For transfers to DBM paper, RNA samples were treated with glyoxal in DMSO and electrophoresed in agarose gels as described by McMaster and Carmichael (1977). Up to 100 μg RNA were loaded per slot (5 mm × 9 mm) in a 7 mm thick vertical 0.8% agarose gel. After electrophoresis, the gel was treated for 20 min in 50 mM NaOH. ABM paper from Enzo Biochemicals, Inc. was diazotized to DBM paper, and the RNA was transferred to it by the procedure of Alwine et al. (1977). An aliquot of each glyoxalated RNA sample was electrophoresed on a separate gel. The gel was treated for 20 min in 50 mM NaOH, 20 min in 0.5 M ammonium acetate and stained for 1 hr in 0.1 M ammonium acetate, 1 μg/ml ethidium bromide.

Preparation of DNA

Various cloned rDNA fragments (Dawid et al., 1978) were prepared from plasmids as described by Wellauer et al. (1976). A number of ribosomal insertion DNA fragments derived from clone Dmra56 were subcloned in the vector pMB9 or pBR322 (Bolivar et al., 1977) following procedures described previously (Wellauer et al., 1976). E. coli HB101 colonies transformed by recombinant plasmids were screened by the procedure of Grunstein and Hogness (1975). All cloning was done under EK1/P2 conditions in accordance with the National Institutes of Health Guidelines for Experiments with Recombinant DNA.

Restriction endonuclease digests of DNA were done according to the instructions of the enzyme manufacturers (Bethesda Research Labs and New England Biolabs), and DNA fragments were separated by electrophoresis in agarose gels. DNA fragments were blotted to nitrocellulose filters by the procedure of Southern (1975). DNA fragments were extracted from agarose gels as described by Tabak and Flavell (1978). Plasmids and DNA fragments were labeled with ^{32}P by the nick-translation reaction as described by Rigby et al. (1977), except that the incubation mixture was extracted with phenol before chromatography on Sephadex. The single-stranded size of nick-translated DNA was measured by electrophoresis in alkaline gels (McDonell, Simon and Studier, 1977).

Hybridizations

Prior to hybridization, DBM paper with bound RNA was treated overnight at 42°C in 2 mg/ml sheared denatured salmon DNA, 50% formamide, 5 × SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 1% glycine and 0.5% SDS. The filters were rinsed in water and hybridized in sealed plastic bags for up to 6 days at 42°C in the above mixture without glycine and with up to 5 × 10^6 cpm/ml of ^{32}P-labeled DNA. The specific activities are given in the figure legends. The filters were washed 4 times at 42°C in 50% formamide, 5 × SSC and 0.5% SDS over a period of 10–15 hr, and 3 times at 42°C in 2 × SSC, 0.5% SDS.

Liquid hybridizations were performed as follows. A fresh ^{32}P-labeled DNA probe (0.5–1.5 × 10^8 cpm/μg) was passed over a Sepharose 6B column in 50 mM NaOH, 1 mM EDTA, and molecules smaller than 200 nucleotides were removed. Foldback molecules were removed from the probe on hydroxylapatite as described by Britten, Graham and Neufeld (1974). Nuclear and cytoplasmic RNA, prepared as described above, were used at concentrations up to 5.3 mg/ml and 9.5 mg/ml, respectively. Samples of 10 μl or 20 μl, in sealed capillaries, in 0.4 M sodium phosphate (equimolar mixture of NaH_2PO_4 and Na_2HPO_4), 5 mM EDTA were denatured for 3 min in boiling water, incubated at 68°C for times ranging from 4 min to 28 hr and frozen in a mixture of dry ice and ethanol. Hybridizations to the external transcribed spacer fragment were performed at 60°C because of its low G+C content. The following treatment of the samples has been modified from the procedure of Galau et al. (1976). Samples were thawed, diluted 10 fold into 10 μg/ml denatured salmon DNA and divided into two parts. One half was adjusted to 0.25 M sodium phosphate and incubated 1 hr at room temperature with 10 μg/ml pancreatic RNAase. RNA-DNA hybrids were shown to resist this treatment. The second half was diluted to 20 mM sodium phosphate and incubated 6–10 hr at 45°C with 50 μg/ml pancreatic RNAase to destroy hybrids. It was shown that over 95% of a reassociated DNA probe resisted this treatment. All samples were then adjusted to 0.12 M sodium phosphate, 0.2% SDS, applied to hydroxyapatite columns at 60°C and fractionated as described by Britten et al. (1974). Nuclear RNA and cytoplasmic RNA samples did not exceed 5.4 μg RNA and 10 μg RNA per ml hydroxyapatite, respectively. The values for reassociated DNA (low salt RNAase treatment) were subtracted from the values for total reassociation (high salt RNAase treatment). Results were expressed in equivalent Rot values by correcting for the acceleration due to a salt concentration higher than 0.12 M sodium phosphate (Britten et al., 1974).

Sandwich hybridizations were performed as described by Dunn and Hassell (1977), with the following modifications. The hybridization with RNA was in sealed plastic bags for 36 hr at 37°C in 50% formamide, 5 × SSC, 0.5% SDS, 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin. Ficoll, polyvinylpyrrolidone and bovine serum albumin were added from a 10 fold concentrated solution after passage over a column of agarose 5′-(p-aminophenyl phosphoryl) uridine 2′(3′) phosphate (Miles Yeda) and storage at −20°C. Filters were washed for 12 hr at 37°C with several changes of 50% formamide, 5 × SSC, 0.5% SDS. Some control filters were washed 3 times in 1 × SSC, treated for 1 hr at 37°C with 20 μg/ml RNAase and washed again in 50% formamide, 5 × SSC, 0.5% SDS. Filters were then hybridized in sealed plastic bags for 36 hr at 37°C with ^{32}P-labeled DNA in the same buffer as that used for the RNA hybridization, and washed in the same way. The RNA hybridization mixture was recovered after the first 36 hr incubation, incubated at 37°C during the remaining 60 hr of sandwich hybridization and washes and electrophoresed in an agarose gel in the presence of 5 M urea. There was no apparent degradation of the RNA compared to unhybridized RNA.

Hybridized filters were dried, covered with Saran-Wrap and exposed to X-ray film. When necessary, films were preactivated and intensifying screens were used as described by Laskey and Mills (1977).

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